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The genome of the southern short-horned tree dragon *Acanthosaura meridiona* Trivalairat, Sumontha, Kunya & Chaingkul, 2022 (Squamata, Draconinae) was analyzed using classical and molecular techniques to identify and study its chromosomal and repetitive elements

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Abstract. The cytogenetics of the southern short-horned tree dragon (*Acanthosaura meridiona*) are not reported yet. This study describes the karyotype of *Acanthosaura meridiona* Trivalairat, Sumontha, Kunya & Chaingkul, 2022 from southern Thailand. We using Giemsa staining, Ag-NOR banding, and fluorescence in situ hybridization (FISH) techniques using microsatellites $d(CA)_{15}$, $d(TA)_{15}$, $d(CGG)_{10}$, and $d(CAA)_{10}$ probes to analyze the chromosome. The karyotype of the *A. meridiona* is 2n = 34 chromosomes (fundamental number of 46), of which 5 pairs were large metacentric chromosomes, 2 pairs small metacentric chromosomes, and 20 microchromosomes (chromosome formula: $2n=34=L^m_{10}+S^m_4+20mi$). There are no sex differences in karyotypes between males and females. The NORs loci were on pair 5 of the large metacentric macrochromosomes. The FISH technique showed $d(CA)_{15}$ and $d(CGG)_{10}$ repeats on specific regions microchromosomes, while signals of $d(TA)_{15}$ and $d(CAA)_{10}$ repeats interspersed on macro- and microchromosomes. This study is significant for enhances our comprehension of the evolutionary mechanism of agamid lizards and promotes the conservation of biodiversity in tropical rainforests.

Keywords: *Acanthosaura meridiona*, chromosome marker, fluorescence in situ hybridization (FISH), microsatellite pattern, Draconinae.

INTRODUCTION

The agamid lizards belonging to the genus *Acanthosaura* Gray, 1831, possess spinose scales with spines on heads and above eyes, along with a prominent spiky crest down their spine (Grismer 2011). All of these species are active during the day and live in trees of Southeast Asia's forested areas, including Myanmar, Thailand, Cambodia, Laos, Vietnam, Yunnan, the Indochinese and Thai-Malay Peninsula, Sumatra, and the Anambas and Natunus Archipelagos (Ananjeva et al. 2008; Manthey 2008; Grismer 2011). The genus *Acanthosaura* currently contains 20 species (Ananjeva et al. 2020; Liu et al. 2020; Trivalairat et al. 2022; Liu et al. 2022; Uetz and Hallermann. 2024).

Currently, there are seven species of *Acanthosaura* in Thailand, namely, *A. armata, A. aurantiacrista, A. cardamomensis, A. crucigera, A. lepidogaster, A. meridiona*, and *A. phuketensis* (Uetz and Hallermann. 2024). *Acanthosaura meridiona* is present in Trang Province, Krabi Province, Nakhon Si Tammarat Province, Songkhla Province, Surat Thani Province, Satun Province, Thailand. *Acanthosaura meridiona* is similar to *A. crucigera* and was previously regarded as an identical species. Wood et al. (2010) found that the southern population of *A. crucigera* exhibited different characteristics that were not present in the true *A. crucigera* population from western Thailand. Nevertheless, *A.* cf. *crucigera* from the southern population has undergone separation into *A. meridiona* (Trivalairat et al. 2022), with the Phuket mountain range acts as a barrier separating the two species. *Acanthosaura* is possible that the extent of variety within this genus is still underestimated. Therefore, cytogenetic research on agamid lizards must achieve greater precision in species differentiation.

Information about karyotypes in *Acanthosaura* only concerns one report in *A. armata* with conventional technique. The karyotypes of Draconinae vary from 2n=32 to 2n=46, with both macrochromosomes and microchromosomes, and absence of sex chromosomes (Ota and Hikida 1989; Sharma and Nakhasi 1980; Li et al. 1981; Ota 1988; Solleder and Schmid 1988; Kritpetcharat et al. 1999; Diong et al. 2000; Ota et al. 2002; Singh and Banerjee 2004; Zongyun et al. 2004; Patawang et al. 2015).

This study looks at the cytogenetic points of view that constitute useful tools of genetic sex chromosome systems, different evolutionary lineages and to delineate evolutionary trends in a great number of taxa (Mezzasalma et al. 2021 and Mezzasalma et al. 2024). This paper first describes *Acanthosaura meridiona*'s chromosomal features, using conventional staining, Ag-NOR banding, and fluorescence in situ hybridization techniques.

MATERIALS AND METHODS

Five adult male and five female specimens of barred gliding lizard (*Acanthosaura meridiona*) were collected from Ban Wang Sai, Mae Wat subdistrict, Than To District, in Yala Province, Thailand. The agamid lizards were transferred to the laboratory and identified according to the morphological criteria (Chan-Ard et al. 2015; Das 2015). Experiments were performed in accordance with ethical protocols, as approved by the Ethics Committee of Prince of Songkla, Pattani Campus (Ref.AI001/2024).

Chromosomes were directly prepared *in vivo* (Patawang et al. 2018) as follows. Metaphasic and meiotic chromosomes were obtained from bone marrow and testis, according the colchicine-hypotonic-fixation-air drying technique (provide references). The chromosomes were stained with 20% Giemsa's for 30 minutes, Ag-NOR staining was conducted according to Howell and Black (1980). Chromosomal checks were performed on mitotic metaphase cells under light microscope.

FISH experiments were performed with microsatellite sequences, specifically $(TA)_{15}$, $(CA)_{15}$, $(CAA)_{10}$, and $(CGG)_{10}$ using high stringency conditions (Yano et al. 2017). The sequences were directly labeled by Cy3 at the 5'end (Sigma, St. Louis, MO, USA) as described by Kubat et al. (2008). FISH was performed under stringent conditions and hybridization in a moist chamber at 37 °C overnight (Sassi et al. 2023). Chromosomes were counterstained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1.2 μg/ml) mounted in antifade solution (Vector, Burlingame, CA, USA,) (Aiumsumang et al. 2021; Patawang et al. 2022; Prasopsin et al. 2022; Thongnetr et al. 2022a).

At least 20 metaphase spreads per individual were analyzed to confirm the diploid number, karyotype structure, NORs and FISH data. Chromosomes were classified according to centromere position as metacentric (m), submetacentric (sm), acrocentric (a), and telocentric (t) (Turpin and Lejeune 1965). For the chromosomal arm number (NF; fundamental number); m, sm and a were scored as bi-armed while t as mono-armed. The microchromosomes are chromosomes that are 5 times less long than the largest pair of chromosomes (Patawang et al. 2016; 2017; 2018).

RESULTS AND DISCUSSION

Mitotic chromosome features from Giemsa staining

Agamidae includes 585 species, of which 94 have been karyologically investigated (Mezzasalma et al. 2024). Karyotypes are discontinuous with a variable chromosome number of macro- and/or micro-chromo-

Table 1. Mean length of short arm chromosome (Ls), length of long arm chromosome (Ll), length of total chromosomes (LT), relative length (RL), centromeric index (CI), and standard deviation (SD) from 20 metaphases of male and female of the southern short-horned tree dragon (*Acanthosaura meridiona*) 2n=34.

Chro. Pair	Ls (μm)	Ll (μm)	LT (μm)	RL±SD.	CI±SD.	Chro.	
						Size	Type
1	2.022	2.772	4.794	0.165±0.014	0.575±0.029	Large	metacentric
2	1.634	1.736	3.371	0.117±0.006	0.517±0.021	Large	metacentric
3	1.411	1.794	3.205	0.110±0.007	0.556±0.031	Large	metacentric
4	1.371	1.684	3.055	0.106±0.005	0.551±0.026	Large	metacentric
5*	1.270	1.509	2.779	0.096±0.004	0.543±0.025	Large	metacentric
6	1.059	1.136	2.195	0.076±0.003	0.516±0.021	Small	metacentric
7	0.982	1.082	2.064	0.071±0.004	0.524±0.022	Small	metacentric
8	0.000	0.813	0.813	0.029±0.002	1.000±0.000	microchromosome	
9	0.000	0.762	0.762	0.027±0.003	1.000±0.000	microchromosome	
10	0.000	0.761	0.761	0.027±0.005	1.000±0.000	microchromosome	
11	0.000	0.762	0.762	0.027±0.003	1.000±0.000	microchromosome	
12	0.000	0.699	0.699	0.024±0.003	1.000±0.000	microchromosome	
13	0.000	0.679	0.679	0.024±0.003	1.000±0.000	microchromosome	
14	0.000	0.632	0.632	0.022±0.002	1.000±0.000	microchromosome	
15	0.000	0.573	0.573	0.020±0.003	1.000±0.000	microchromosome	
16	0.000	0.540	0.540	0.019±0.003	1.000±0.000	microchromosome	
17	0.000	0.469	0.469	0.016±0.003	1.000±0.000	microchromosome	

* = NORs bearing chromosomes, Chro. = Chromosome.

somes, namely macrochromosomes ranging from 10 to 28, and microchromosomes from 0 to 24 (Mezzasalma et al. 2024). In Draconinae, based on 21 species reports, karyotypes range from 2n=32- to 46 (Table 2). However, so far the present study first reports the karyotype of *Acanthosaura meridiona*, loci of NORs, and by Fluorescence in situ hybridization the distribution of $(TA)_{15}$, $(CA)_{15}$, $(CAA)_{10}$, and $(CGG)_{10}$ microsatellites.

The results revealed that the chromosome number of *A. meridiona* was 34 (14 macrochromosomes, and 20 microchromosomes). The karyotype comprised ten large metacentric chromosomes, four small metacentric chromosomes, and 20 microchromosomes (Table 1 and Figure 1). This result differs with from that of *A. armatus* of 2n=32 with 12 metacentric macrochromosomes, 20 microchromosomes. The fundamental number (NF) of *A. meridiona* and *A. armatus* was 46 and 44, respectively. It is possible that the different macrochromosome numbers may have been caused by an event of tandem fusion and centromere deletion involving the chromosome number and NF variation. A similar process of autonomous reduction in total chromosomal number through autosome translocation has been reported in other lizard families, including Anguidae, Scincidae, Iguanidae, Gekkonidae, and Phrynosomatidae (Adegoke and Ejere 1991; Trifonov et al. 2015).

There is no evidence of differentiated sex chromosomes in this species which agreeable with all species of Draconinae (Ota and Hikida 1989; Sharma and Nakhasi 1980; Li et al. 1981; Ota 1988; Solleder and Schmid 1988; Kritpetcharat et al. 1999; Diong et al. 2000; Ota et al. 2002; Singh and Banerjee 2004; Zongyun et al. 2004; Patawang et al. 2015). Squamates exhibit a considerable degree of variability in their chromosome sex determination systems. Various families exhibit diverse sex-chromosome systems, which can be either simple or multiple, and include either male (XX/XY) or female (ZZ/ZW) heterogamety. These systems encompass all hypothesized stages of heterogametic sex chromosomes, including homomorphic and pseudo-autosomal to heteromorphic and completely heterochromatic chromosomes (Alam et al. 2018 and Mezzasalma et al. 2021).

Nucleolar organizer region from Ag-NOR banding

Ag-NOR banding, a species-specific marker, primarily identifies karyotypes. Silver staining, on the other hand, only detects the nucleolar organizer areas that are actively involved in transcription (Silva et al. 2008). The improvement of the Ag-NOR staining method has been very important in comparing NOR variation because it

Table 2. Comparative chromosome studies of subfamily Draconinae.

Species	2n	Karyotype	NOR	Locality	References
Acanthosaura armata	32	12m+20mi	-	Malaysia	Ota et al. (2002)
A. meridiona	34	14m+20mi	5qter	Thailand	This study
Bronchocela cristatella	34	14m+20mi	-	Singapore	Ota et al. (2002)
	34	12m/sm+22mi	2qter	Asia	Solleder and Schmid (1988)
Calotes emma alticristatus	34	12m/sm+22mi	2qter	Asia	Solleder and Schmid (1988)
	34	12m/sm+22mi	-	Thailand	Kritpetcharat et al. (1999)
	34	12m+22mi	-	Malaysia	Ota et al. (2002)
	34	-	-	India	Singh and Banerjee (2004)
C. jerdoni	34	12m/sm+22mi	-	India	Sharma and Nakhasi (1980)
	34	-	-	India	Singh and Banerjee (2004)
C. mystaceus	34	12m/sm+22mi	2qter	Asia	Solleder and Schmid (1988)
	34	12m/sm+22mi	-	Thailand	Kritpetcharat et al. (1999)
	34	-	-	India	Singh and Banerjee (2004)
	34	10m+2m+22mi	2qter	Thailand	Patawang et al. (2015)
C. versicolor	34	12m/sm+22mi	2qter	Asia	Solleder and Schmid (1988)
	34	12m/sm+22mi	-	Thailand	Kritpetcharat et al. (1999)
	34	12m+22mi		Singapore	Ota et al. (2002)
	34	12m/sm+22mi	2qter	Thailand	Patawang et al. (2015)
C. vultuosus	32, 34	-	-	India	Singh and Banerjee (2004)
	34	12m/sm+22mi	-	India	Sharma and Nakhasi (1980)
Draco cornutus	34	16m+18mi	-		
D. haematopogon	34	16m+18mi	-	Malaysia	Ota and Hikida (1989)
D. quinquefasciatus	34	16m+18mi	-		
Diploderma splendidum	34	12m+22mi	-	China	Zongyun et al. (2004)
D. swinhonis	36	10bi+26a	-	Central Taiwan	Ota (1988)
	40	6bi+34a	-	Central Taiwan	
	46	46a	-	Northern Taiwan	
Gonocephalus chamaeleontinus	42	22m+20mi	-		
G. liogaster	42	22m+20mi	-		
G. bellii	42	22m+20mi	-	Malaysia	Diong et al. (2000)
G. grandis	42	22m+20mi	-		
G. robinsonii	32	12m+20mi	-		
Japalura variegata	34	-	-	India	Singh and Banerjee (2004)
J. varcoae	34	12m+22mi	-	China	Li et al. (1981)
Ptyctolaemus gularis	34	12m/sm+22mi	-	India	Sharma and Nakhasi (1980)

Note: 2n: diploid chromosome number, m: metracentric, sm: submetracentric, a: acrocentric, bi: biarms, mi: microchromosome, and qter: long arm of chromosome.

lets us find the metaphase chromosomal locations that are linked to NOR. The present study, the chromosome markers of *A. meridiona* observable NORs on the telomeric region of large metacentric macrochromosome pair 5th (Figure 2). Similarly, the previous report of NOR position in Draconinae was located on telomeric region of q-arm in 4 species of *Calotes* consisting of *C. cristatellus*, *C. emma*, *C. mystaceus*, and *C. versicolor* (Solleder and Schmid 1988; Patawang et al. 2015). However, findings from both traditional and molecular cytogenetics suggest that the location of NOR loci on microchromosomes is usually thought of as an ancestral trait in most families and genera (Mezzasalma et al. 2021; Waters et al. 2021; Deakin and Ezaz 2019).

Microsatellite pattern

Microsatellites, also known as simple sequence repeats (SSRs), are short DNA sequences consisting of

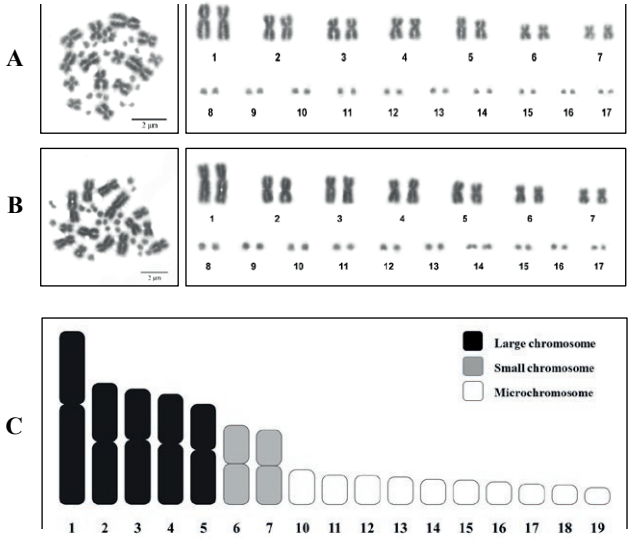

Figure 1. Metaphase plates and standardized karyotypes of male (A.), female (B.) and Idiogram (C.) of the southern short-horned tree dragon, *Acanthosaura meridiona*, 2n=34 by conventional staining.

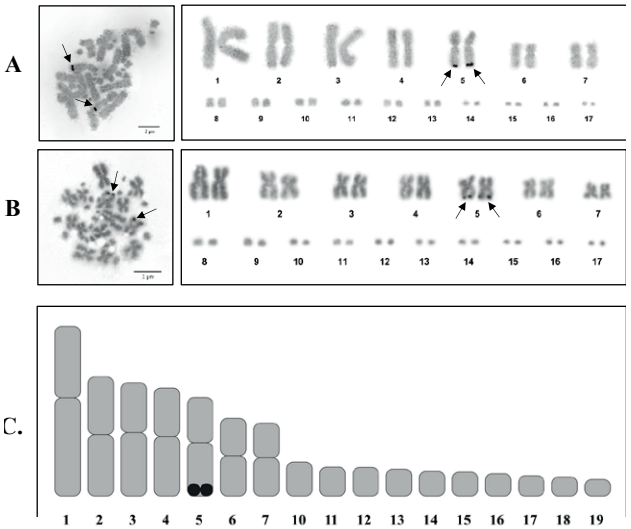

Figure 2. Metaphase plates and standardized karyotypes of male (A.), female (B.) and Idiogram (C.) of the southern short-horned tree dragon, *Acanthosaura meridiona*, 2n=34 by Ag-NOR banding, arrows indicate NORs.

1–6 base pairs. They are distinguished by the presence of repetitive units, which can range from 4 to 40 repeats in a sequence (Tautz and Renz 1984; Ellegren 2004; Chistiakov et al. 2006). They appear either dispersed or clustered in euchromatin and heterochromatin regions, widely distributed across eukaryotic genomes. They show

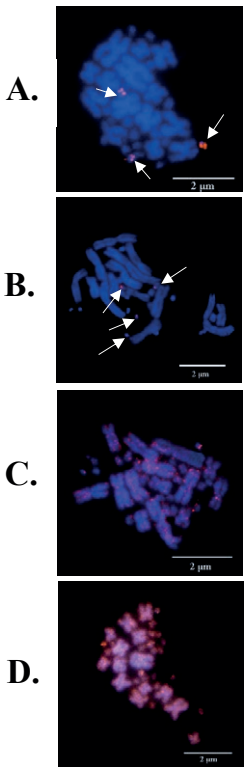

Figure 3. Metaphase plates and hybridization patterns with microsatellite probes d(CA)$_{15}$ (A.), d(CGG)$_{10}$ (B.), d(TA)$_{15}$ (C.), and d(CAA)$_{10}$ (D.) (red signals) on metaphase plates of the southern short-horned tree dragon, *Acanthosaura meridiona*, 2n=34, chromosomes were counterstained with DAPI (blue).

Table 3. The hybridization patterns with microsatellite probes d(CA)$_{15}$, d(CGG)$_{10}$, d(TA)$_{15}$, and d(CAA)$_{10}$ of the southern short-horned tree dragon (*Acanthosaura meridiona*).

Probe	Signal
d(CA)$_{15}$	Pair 13 & 15
d(CGG)$_{10}$	Pair 15 & 16
d(TA)$_{15}$	Throughout genome (weak)
d(CAA)$_{10}$	Throughout genome (strong)

a significant variation in the number of copies of genetic material (Ellegren 2004). Microsatellite repeat patterns of *A. meridiona* indicated the presence of specific regions on microchromosomes, including pair 13 and 15 with d(CA)$_{15}$ repeats, and pair 15 and 16 with d(CGG)$_{10}$ repeats. While d(TA)$_{15}$ and d(CAA)$_{10}$, showed cumulative signals dispersed throughout the chromosomes (Table 3, Figure 3). The microsatellite loci exhibited a significant level of evolutionary advancement. Thus, it is common for many species to have diverse patterns of repeated sequences. Most of them and scattered them

across the genome (Thongnetr et al. 2019; 2022a; 2022b; Khawporntip et al. 2024). However, in certain species, they may localize into specific regions (Srikulnath et al. 2009; Alam et al. 2021). Interestingly, signals of the $d(CA)_{15}$ probe specifically is on a chromosome of pair 13, a finding that is unclear and remains unexplained. This suggests that using Fluorescence in situ Hybridisation (FISH), the process of mapping cDNA or BAC clones to the chromosomes of southern short-horned tree dragon has successfully addressed certain constraints (O'Meally et al. 2009; Alföldi et al. 2011; Srikulnath et al. 2015; Young et al. 2013; Deakin et al. 2016; Badenhorst et al. 2015). By integrating data from several species, one can obtain intra-sequence information that enhances our understanding of the evolution of chromosome in lizards.

In conclusion, we first present the karyotype, NORs and microsatellite $d(CA)_{15}$, $d(TA)_{15}$, $d(CGG)_{10}$, and $d(CAA)_{10}$ patterns on the chromosomes of the southern short-horned tree dragon. *Acanthosaura meridiona* has 2n=34 chromosomes (14 macrochromosomes, and 20 microchromosomes), NF=46. The karyotype consisting of 5 pairs of large metacentric chromosomes, 2 pairs of large metacentric chromosomes, and 20 microchromosomes. NORs were located on the telomeric region of large metacentric macrochromosome pair 5^{th}. Microsatellite repeat patterns indicated the presence of specific regions on microchromosomes, including pair 13 and 15 with $d(CA)_{15}$ repeats, and pair 15 and 16 with $d(CGG)_{10}$ repeats. While $d(TA)_{15}$ and $d(CAA)_{10}$, showed cumulative signals dispersed throughout the chromosomes. This study is valuable for improving our understanding of the evolutionary process of agamid lizards and advocating for biodiversity protection in tropical rainforests. Moreover, we suggest that more species be studied using cytogenetics and that techniques be investigated further to gain a deeper understanding of chromosomal diversity and evolution within this genus.

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